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Identification of *Salmonella* Strains of Phyllosphere Food Poisoning by Melt Curve Analysis: *In Silico* approach

Rahul Prakash[1]*, S. N. Jha[2], V.K.Mishra[3]

[1]Department of Biotechnology, L.N.Mithila University, Darbhanga-846004, Bihar, India

[2]Microbial Genetices and Applied Biotech Lab, L.N.Mithila University, Darbhanga-846004, Bihar, India

[3]Department of Biotechnology, Doon (P.G.) Paramedical College, Dehra Dun-248001,Uttarakhand, India

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ABSTRACT

During last few decades, there have been increased incidences of outbreak of diseases due to consumption of fresh vegetables and fruits contaminated with human pathogens. Such threats warrant rapid detection test. The standard method of diagnosis relies on culture-plate and serological methods which lack discrimination and are time consuming having several drawbacks, inconsistency and are less efficient. We applied bioinformatics approaches to develop of a real- time PCR simulation for detecting Salmonella serovars which are involved in most disease outbreaks associated with phyllosphere. Salmonella enterica subspecies enterica (designated as S. enterica) are common in plants, on surface as well as present internally in tissues. Though more than 2500 serovars of Salmonella enterica are known but the reports of serovars colonizing in plants are limited. Nucleotide sequence variation in target genes, viz. PurE, SucA, hisD, hivA and fliC were used in in silico to differentiate Salmonella serovars. A large number of reference sequences of target genes were retrieved from NCBI, and common conserved region were used for development of multiplex primer design using muPlex. Primer thermodynamic properties and secondary structure were assessed using Beacon designer. Sequences were truncated to remove sequences outside of the region bounded by the primers. We performed in silico DNA melting simulations with several Salmonella serovars using the programs umelt, and tested the utility of the programs for assay design, which will save time and cost of in vitro testing several multiple primers in RT PCR.

Corresponding Author:

Rahul Prakash
Department of Biotechnology,
L.N.Mithila University,
Darbhanga-846004,
Bihar, India.

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1. INTRODUCTION

Salmonella is a major foodborne pathogenic bacterium. Salmonellosis is responsible for large numbers of infections in both humans and animals [1]. *Salmonella* strains are not detectable in certain clinical samples that contain small numbers of organisms [2]. The *Salmonella* genus consists of 2 species: *S. bongori* and *S. enterica*. The latter includes 6 subspecies: *S. enterica ssp. houtenae, arizonae, diarizonae, enterica, salamae* and *indica*. *S. enterica* spp. *enterica* includes the human pathogenic *Salmonella* and consists of more than 2000 serovars including *Typhimurium* (of which more than 500 phage types are recognized), *Typhi*, *Dublin*, *Enteritides*, *Montevideo*, *Newport* etc.*Salmonella* spp. are resilient bacteria and can adapt to extreme environmental conditions [3]. *Salmonellae* have been isolated from many types of raw fruits and vegetables. The inspection of

food for the presence of *Salmonella* has become routine all over the world. Due to the low infective dose of *Salmonella*, methods for its detection are required to prove the presence of one cell in a defined food sample. Cultural methods for *Salmonella* detection involve a no selective pre-enrichment, followed by selective enrichment and plating on selective and diagnostic agars. Suspect colonies are confirmed biochemically and serologically; the complete test requires three to four days to obtain a negative result and up to seven days to get a confirmed positive result [4].

A number of rapid methods for the detection of *Salmonella* in foods have been developed, including electrical techniques, immunoassays and nucleic acid probe analyses [5, 6, 7, 8]. However, there are still problems with their sensitivity and specificity. Several PCR assays have been developed by targeting various *Salmonella* genes, such as *invA* [9, 10], 16S rRNA [11], *agfA* [12], and *viaB* [13], and virulence-associated plasmids [14, 15]. Guo *et al*. (2000) developed a PCR to detect *S. Montevideo* in artificially infected tomatoes after enrichment, using two pairs of primers to amplify the hilA gene [11].Thus, rapid and sensitive methods for detecting *salmonellae* are in great demand in order to assure produce safety. High Resolution Melting (HRM) is a simple, rapid and low cost genotyping method [16]. Its advantage is the fact that PCR amplification and melting curve analysis are performed within the same tube or plate, without any post-PCR processing. This feature is particularly important for a routine diagnostic setting.

Since HRM is based on thermodynamic differences between DNA fragments, it has been used in particular for scanning of heterozygous sequences. However, in its original form, discrimination between homozygous genotypes is more difficult, because the difference between homozygous sequence melting profiles is usually merely represented by a slight shift in the melting temperature (Tm), but not by a change of the melting curve profile [17]. Therefore, HRM has been adapted for analysis of polymorphic SNPs via PCR amplification of small amplicons. Such a reduction of the amplicon length results in a broader divergence between melting profiles and increases the sensitivity of the technique, which then could be used not only for scanning, but also for accurate genotyping. Moreover, differences between homozygous wild type and homozygous mutant DNA fragments are thus more apparent [18].

Although reviews and reports on the use of HRM for mutation scanning and genotyping were published previously [16], there is no report on diagnostic validation of this technique as required by OECD and/or ISO guidelines .In this manuscript, we have evaluated *in silico* DNA melting simulations with several *Salmonella* serovars using the POLAND program and umelt program, and tested the utility of the programs for assay design, which will save time and cost of *in vitro* testing several multiple primers in RT- PCR.

2. METHODOLOGY

2.1 Target Genes

Five different housekeeping genes were selected as a target for identification of different strains of salmonella from the genome of salmonella serovars .The genes used are purE (phosphoribosylaminoimidazole carboxylase), sucA (alpha ketoglutarate dehydrogenase), hisD (histidinol dehydrogenase), hivA (invasion protein regulator) and fliC (flagellin) (Table 1) and their Corresponding sequence were downloaded from GenBank (http://www.ncbi.nlm.nih.gov).

Table 1. Different Target genes used in *Salmonella serovars* differentiation

Serial no.	Target Genes	Description of Target Genes
1	purE(phosphoribosylaminoimidazole carboxylase)	Involved in nucleotide biosynthesis and in particular in purine biosynthesis
2	sucA(alpha ketoglutarate dehydrogenase)	Role in the citric acid cycle
3	hisD (histidinol dehydrogenase)	Biosynthesis of histidine in bacteria, fungi, and plants
4	hivA(invasion protein regulator)	Regulates transcription
5	fliC (flagellin)	Structural gene/help in locomotion

2.2 Melting simulations

GenBank accession numbers for DNA sequences used in the in *in silico* simulations are: CAAW01000170.1 (*S. enterica* subsp. *enterica serovar Typhi str.* J185), AFCM01000213.1 (*S.enterica subsp. enterica serovar Give str*. S5-487), AFYI01000002.1 (*S.enterica subsp. enterica serovar Infantis* str. SARB27), AFCT01001240.1 (*S.enterica subsp. enterica serovar Rubislaw* str. A4-653), CAAY01001042.1(*S.enterica subsp. enterica serovar Typhi* str. AG3), AFCJ01001318.1 (*S.enterica subsp. enterica serovar Alachua* str. R6-377)**,** AFCX01001122.1 (*Salmonella enterica subsp. enterica serovar Wandsworth* str. A4-580 Contig1122), AFCU01001099.1 (*Salmonella enterica subsp. enterica serovar Senftenberg* str. A4-543), AFCR01000465.1 (*S.enterica subsp. enterica serovar Mississippi* str. A4-633), AFCK01001128.1 (*S.enterica subsp. enterica serovar Baildon* str. R6-199), CAAS01000662.1 (*S.enterica subsp. enterica serovar Typhi* str. E01-6750), CAAT01000074.1 (*S.enterica subsp. enterica serovar Typhi* str. E02-1180), AFCW01001647.1 (*S.enterica subsp. enterica serovar Urbana* str. R8-2977), CAAX01001539.1 (*S.enterica subsp. enterica serovar Typhi* str. M223). Where required, sequences were truncated to remove sequences outside of the region bounded by the primers. Melting simulations were performed using the UMELT program and the POLAND programme (for both programmes Default settings were taken). Critical output data relevant to this study were the "differentiated hypochromicity at 260 and 282 nm (dA/dT) vs. temperature" plot and the first and second order reaction stack indices for temperature pf 50% probability for POLAND and the melt map for each sequence (base position versus predicted *T*m) determined by umelt.

2.3 Primer designing and Thermodynamic properties

Designing of Primers using *muPlex (multi-objective multiplex PCR assaydesign)software*.[http://apps.diatomsoftware.com/muplex/html/MuPlex.html] and Primer properties obtained from free web version of Beacon Designer (http://www.premierbiosoft.com/jsp/marketing/FreeToolLogin.jsp?PID=1) (Table 2).

Table 2. Primer designing and properties **(**Designing of Primers using *muPlex software and* properties obtained from free web version of Becon Designer).

Target Gene	Primer	Tm	GC%	GC Clamp	Cross Dimer (ΔG)	Self Dimer (ΔG)	Hairpin (ΔG)
purE	F-GTACGCTGGCGATCGGTA(18bps)	55.75	61.11	3	-2.9	-6.7	-0.3
	R-AATGCGCTGATGCAGTTC(18bps)	52.96	50	3	-2.9	-5.2	-2.0
sucA	F- CGCAGGTAGTGATTGACCAG(20bps)	55.38	55	1	-4.3	-1.5	-1.5
	R-TCATAGCCATGCGGCAAC (18bps)	54.59	55.56	1	-4.3	-2.4	-2.4
hisD	F-GTGGATGCGATTACCAGC(18bps)	52.43	55.56	2	-2.9	-1.5	-1.5
	R-GTAAGACATGGTTGGTTCCG(20bps)	53.47	50	2	-2.9	-2.3	0.0
hivA	F-GACAGAGCTGGACCACAATAAT(22bps)	55.57	45.45	0	-1.8	-3.0	0.0
	R- AGCAAACTCCCGACGATG(18bps)	53.84	55.56	0	-1.8	0.0	0.0
fliC	F- TGGCGGTTCAGTCTGCTA(18bps)	54.56	55.56	2	-1.1	-1.1	0.0
	R- CGGTCGATTTCGTTCAGG(18bps)	52.46	55.56	2	-1.1	-3.1	0.0

2.4 Finding intervening sequence

Intervening sequence were find by the use of mega4software (http://www.megasoftware.net/mega4/mega.html).

3. RESULT AND DISCUSSION

Differentiation of PCR products using DNA melting curve analysis was first demonstrated by Ririe *et al* with the double-stranded DNA-specific dye SYBR Green I and has since seen widespread adoption in real-time PCR applications [19]. Melting curve analysis provides immediate practical benefits in real-time PCR, obviating the need for gel electrophoresis by providing a reproducible signature of the amplified DNA sequence that may be used for typing PCR products. Typing is typically achieved by examining the first derivative of the melting

curve and identifying the characteristic "melt peak" (*T*m), which is the temperature at which the rate of fluorescence change (DNA denaturation) is highest and is observed in the raw data as a sudden decrease in fluorescence [20].Genotyping by HRM of small amplicons is a technique associated with high sensitivity and specificity [20]. We were able to prove these observations by discriminating different strains of *salmonella serovars*. Complex melting profiles resulting in multiple melting peaks provide a superior tool for identification of different strains of *salmonella* serovars compared with simple profiles that result in only a single peak. In addition to Tm, both the number of peaks and relative peak heights can be used as additional diagnostic characters to facilitate identification of different strains *salmonella* serovars. Until this study, a systematic method for developing such assays had not been reported. The ability to simply submit a DNA sequence to melting simulation software and generate a useful prediction of the likely real time PCR melting curve profile *in silico* was an interesting and it also obviates the need of gel electrophoresis. A number of programs are available to help design primers pairs and pick target sequence. Primer-Blast, a freeware option for desiging oligonucleotides, is a program developed by NCBI that uses the algorithm primer3. Primer sequences are compared users selected database to ensure they are unique and specific for the gene of interest. Melt peak temperature (T) comparison in 14 serovars of *S.entrica sub sp. Entrica* (Fig 1) and *the* melt map for each sequence/amplicon (base position versus predicted Tm) shows/gives a clear result demonstrate that the melting curves we designed has high specificity in the identification of different strains of *salmonella serovars*. Multiple melting domains in a melting curve provide a rich source of information for typing samples-like *salmonella* (Fig 2).

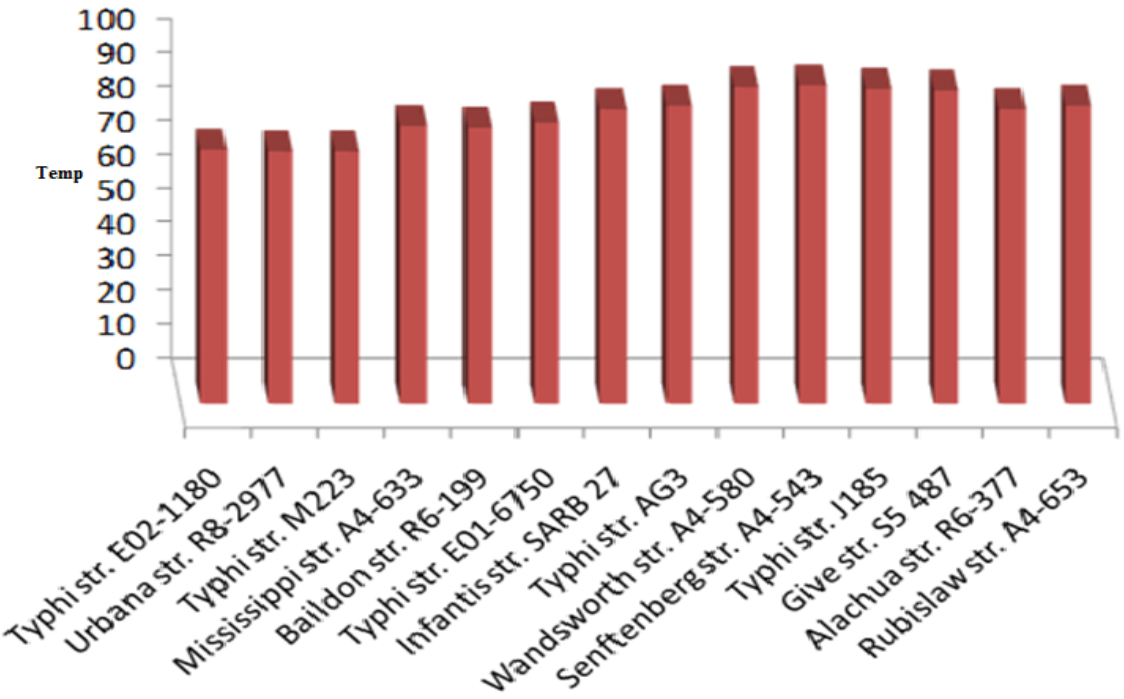

Figure 1.Melt peak temperature (T) comparison in 14 serovars of *S.entrica sub sp. enterica*

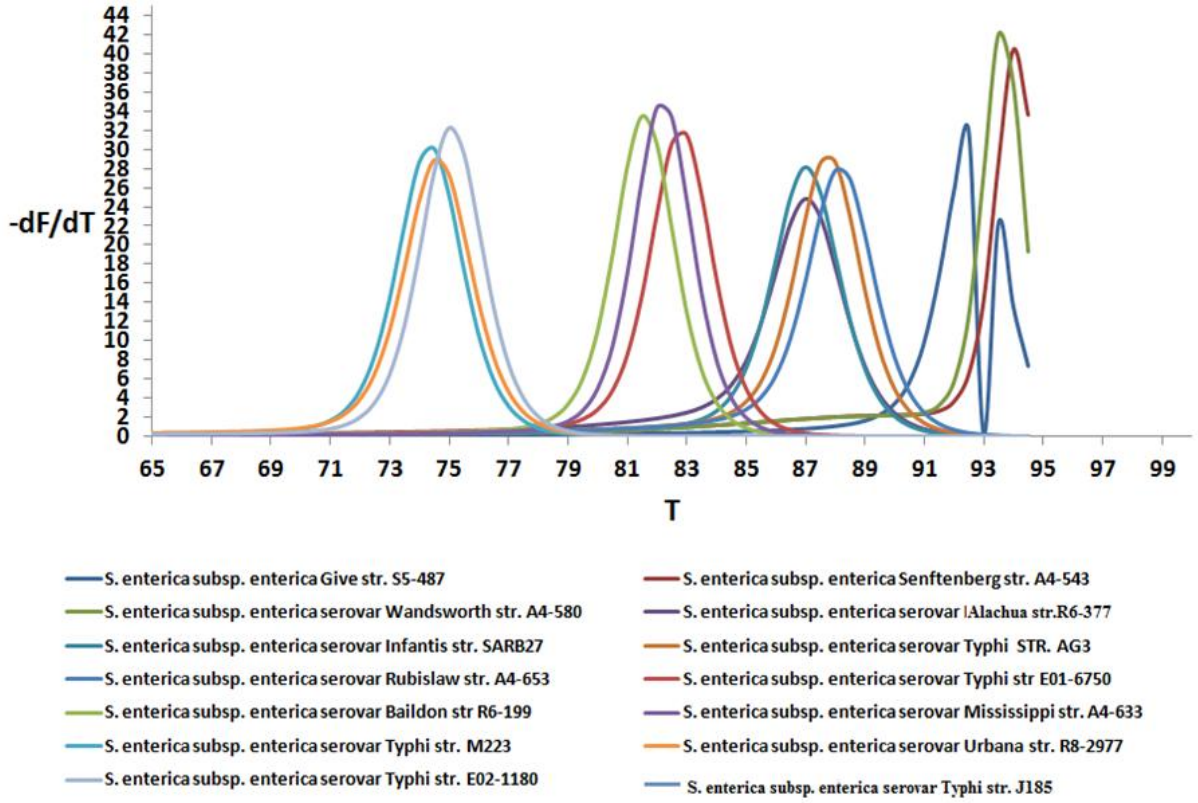

Figure 2. Simulated multiple melting profile of different *S.entrica sub sp.entrica serovars*

4. CONCLUSION

The phyllosphere is both scientifically and economically an important habitat in which to study microbial ecology. Because of the importance of many phyllosphere microbial inhabitants to plant health, there will likely be many practical applications that result from a better understanding of the interactions of microbes with plants and among themselves. This enhanced knowledge may contribute also to our understanding of the ecology of humanpathogenic bacteria on plant surfaces and provide new insights for the development of prevention or control strategies to manage preharvest contamination of crops with enteric pathogens. Computer aided melting simulation provides a gateway to the exploitation of this information. The demonstration that melting curve assays can be designed reliably *in silico* may stimulate more widespread use of whole product melting curve analysis and ultimately lead to dedicated melting curve assay design software.

REFERENCES

[1] Guo X, Chen J, Beuchat LR, Brackett RE. PCR detection of *Salmonella enterica* serotype *Montevideo* in and on raw tomatoes using primers derived from hilA. Appl Environ Microbiol 2000; 66: 5248–5252.

[2] Chiu CH, Ou JT. Rapid identification of *Salmonella* serovars in feces by specific detection of virulence genes, invA and spvC, by an enrichment broth culture-multiplex PCR combination assay. J Clin Microbiol 1996; 34: 2619–2622.

[3] Foster JW, Spector MP. How *Salmonella* survive against the odds. Annual Review of Microbiology 1995; 49:145-174.

[4] Anonymous (1995) Microbiology—General guidance on methods for the detection of *Salmonella.* CSN ISO 6579.

[5] Bennet AR, Greenwood D, Tennant C, Banks JG, Betts RP. Rapid and definitive detection of *Salmonella* in foods by PCR. Lett Appl Microbiol 1998; 26:437–441.

[6] Blivet D, Salvat G, Humbert F, Colin P**.** Development of a new culture medium for the rapid detection of *Salmonella* by indirect conductance measurement. J Appl Microbiol 1998; 84:399–403.

[7] Joosten HMLJ, van Dijck WGFM, van der Velde F. Evaluation of motility enrichment on modified semisolid Rappaport-Vassiliadis medium (MRSV) and automated conductance in combination with Rambach agar for *Salmonella* detection in environmental samples of milk powder factory. Int J Food Microbiol 1994; 22:201–206.
[8**]** Lee HA, Wyatt GM, Bramham S, Morgan MRA. Enzyme-linked immunosorbent assay for *Salmonella typhimurium* in food: Feasibility of 1-day Salmonella detection. Appl Environ Microbiol 1990; 56:1541–1546.
[9] Iida K, Abe A, Matsui H, Danbara H, Wakayama S, Kawahara K. Rapid and sensitive method for detection of *Salmonella* strains using a combination of polymerase chain reaction and reverse dot-blot hybridization. FEMS Microbiol Lett. 1993;114:167–172.
[10] Kimura B, Kawasaki S, Fujii T, Kusunoki J, Itoh T, Flood S J A**.** Evaluation of TaqMan PCR assay for detecting *Salmonella* in raw meat and shrimp. J Food Prot. 1999;62:329–335.
[11] Rahn K, De Grandis S A, Clarke R C, McEwen S A, Galan J E, Ginocchio C, Curtiss III R, Gyles C L. Amplification of an invA gene sequence of *Salmonella typhimurium* by polymerase chain reaction as a specific method of detection of *Salmonella*. Mol Cell Probes. 1992;6:271–279.
[12] Doran JL, Collinson SK, Burian J, Sarlos G, Todd E C D, Munro CK, Kay CM, Banser PA, Peterkin PI, Kay WW. DNA-based diagnostic test for *Salmonella* species targeting agfA, the structural gene for thin aggregative fimbriae. J Clin Microbiol. 1993;31:2263–2273.
[13] Hashimoto Y, Itho Y, Fujinaga Y, Khan AQ, Sultana F, Miyake M, Hirose K, Yamamoto H, Ezaki T. Development of nested PCR based on the ViaB sequence to detect *Salmonella typhi*. J Clin Microbiol. 1995;33:775–777.
[14] Wang RF, Cao WW, Cerniglia CE. A universal protocol for PCR detection of 13 species of foodborne pathogens in foods. J. Appl. Microbiol. 1997; 83:727–736.
[15] Jothikumard N, Griffiths MW. Rapid detection of Escherichia coli O157:H7 with multiplex real-time PCR assays. Appl. Environ. Microbiol. 2002; 68:3169–3171.
[16**]** Reed GH, Wittwer CT. Sensitivity and specificity of single-nucleotide polymorphism scanning by high-resolution melting analysis. Clin Chem 2004;50:1748–54.
[17] Palais RA, Liew MA, Wittwer CT. Quantitative heteroduplex analysis for single nucleotide polymorphism genotyping. Anal Biochem 2005;346(1): 167–75.
[18] Liew M, Pryor R, Palais R. Genotyping of Single-Nucleotide Polymorphisms by High-Resolution Melting of Small Amplicons. Clin Chem 2004; 50: 1156–1164.
[19] Wilhelm J, Pingoud A. Real-time polymerase chain reaction**.** *Chembiochem* 2003, 4(11):1120-1128.
[20] Ririe KM, Rasmussen RP, Wittwer CT. Product differentiation by analysis of DNA melting curves during the polymerase chain reaction**.** *Anal Biochem* 1997, 245(2):154-160.